Journal of Medical Virology 50:16–19 (1996)

Hepatitis E Virus Seroprevalence in Acute Viral Hepatitis in a Developed Country Confirmed by a Supplemental Assay

Juan Antonio Quiroga, Teresa Cotonat, Inmaculada Castillo, and Vicente Carreño

Hepatology Unit, Fundación Jiménez Díaz, Madrid (J.A.Q., T.C., I.C., V.C.); and Fundación para el Estudio de las Hepatitis Virales, (J.A.Q., T.C., I.C., V.C.) Spain

Hepatitis E virus (HEV) infection is prevalent among cases of acute viral hepatitis in young adults in developing countries. HEV infection is not restricted to endemic areas, but would appear to be worldwide in distribution. In order to document the incidence of HEV infection in acute hepatitis cases in a developed country, IgG and IgM anti-HEV antibodies and HEV RNA were tested in 101 Caucasian patients with acute viral hepatitis; 92 of these cases had markers of acute viral hepatitis other than HEV. Forty-seven (46.5%) cases had IgG anti-HEV; IgM anti-HEV and HEV viremia were not detected. As the incidence of anti-HEV was higher than would be expected, the possibility of the occurrence of false positive results was subsequently investigated. Supplemental antibody testing, using a broadly reactive epitope region, reduced the frequency of anti-HEV to 17%. Therefore, supplemental antibody testing confirms the hepatitis E virus seroprevalence in a developed country. Since IgM anti-HEV and HEV viremia were not detected, persons with IgG anti-HEV may be "subclinical HEV cases," or have long-lived antibodies in their circulation.

KEY WORDS: hepatitis E virus (HEV), IgG and IgM anti-HEV, HEV RNA, acute viral hepatitis, supplemental assay

INTRODUCTION

Hepatitis E virus (HEV) is the major etiologic agent of enterically transmitted non-A, non-B hepatitis [Reyes et al., 1990; Tam et al., 1991]. Two distinct HEV isolates were first recognized, and designated as the Burma (B) prototype [Tsarev et al., 1992] and the Mexico (M) [Huang et al., 1992] strains; two other isolates from Pakistan and The People's Republic of China have recently been sequenced [reviewed by Bradley, 1995]. The M strain contains some epitopes which differ from those found in the B strain [Yarbough et al., 1991]. Current immunoassays for the detection of antibodies to HEV (anti-HEV) use a mixture of recombinant proteins derived from open reading frames (ORF) 2 and 3 (encoding HEV structural proteins) from either the B strain alone [Dawson et al., 1992], or from both the B and M strains [Yarbough et al., 1994]. Seroprevalence studies of anti-HEV have shown that hepatitis E accounts for more than 50% of the cases of acute viral hepatitis in young adults in developing countries. However, HEV infection is not restricted to such endemic areas, but would appear to be worldwide in distribution. In Western countries, anti-HEV has been detected in 2–7% of the volunteer blood donors in the United States and Germany [Dawson et al., 1992], and in 0.4–2.1% in the Netherlands [Zaaijer et al., 1993]. In this work, the frequency of HEV infection in acute viral hepatitis in a developed country has been investigated retrospectively.

PATIENTS AND METHODS

Patients

The population studied included 101 Caucasian patients (34 female, 67 male; mean age of 30.4 years, range 14 to 76) born in developed countries (all were from Spain except two who were born in the UK). They were sequential unselected cases who were seen as outpatients in our hospital between 1985 and 1993. The diagnosis of acute viral hepatitis was based on clinical data (jaundice and lack of a previous history of liver disease), ALT values more than 20 times the upper limit of normality, and on serological parameters. Among the 101 patients, 92 had markers of acute viral hepatitis other than HEV. Thus, 45 patients had IgM anti-HAV positive; 16 had IgM anti-HBc and HBsAg positive; seven had markers of acute hepatitis B and D (IgM anti-HBc, HBsAg and IgM anti-HDV positive); 15 had markers of acute hepatitis C (IgG and IgM anti-HCV and HCV RNA

Accepted for publication April 18, 1996.

Address reprint requests to Dr. V. Carreño, Hepatology Unit, Fundación Jiménez Díaz, Av. Reyes Católicos 2, 28040 Madrid, Spain.

positive) [Farci et al., 1991; Chau et al., 1991; Clemens et al., 1992]; six had markers of acute hepatitis B and C; three had markers of acute hepatitis B, C, and D. The remaining nine patients were diagnosed by exclusion criteria as having acute non-A, non-B, non-C (NANBNC) hepatitis (in the absence of markers of acute HAV, HBV, HCV, HDV, Epstein-Barr virus, and Cytomegalovirus infections). This study was conducted in accordance with the Declaration of Helsinki on human experimentation and patients gave informed consent.

Methods

Serum samples were collected within 4 weeks of the onset of symptoms; these were aliquoted and stored at −20°C until use. IgM anti-HAV, IgM anti-HBc, HBsAg, and IgM anti-HDV were determined by commercial assays (Abbott Labs., North Chicago, IL). IgG anti-HCV was tested by third generation ELISA and confirmed by RIBA (Ortho Diagnostic System, Raritan, NJ). Serum HCV RNA was tested by reverse transcription and "nested" polymerase chain reaction (RT-PCR) [Castillo et al., 1994], using primer pairs derived from the 5′ noncoding region of the HCV genome. IgM anti-HCV-core was assayed at 1:10 dilution by ELISA using a synthetic oligopeptide whose sequence was derived from the amino terminal portion of the HCV-core protein as recently described [Quiroga et al., 1995].

IgG anti-HEV was examined by two commercially available immunoassays: assay no. 1, with recombinant proteins SG-3 and 8-5 derived from HEV-ORF2 and ORF3 of the B strain, respectively, in a coated-bead format with samples diluted 1:400 (Abbott Laboratories); and assay no. 2, with recombinant antigens 3-2 (M strain) derived from HEV-ORF2 and 4-2 antigens (B and M strains) derived from HEV-ORF3 in a microtiter plate format with wells coated with HEV antigens and samples diluted 1:20 (Genelabs Technologies Inc., Redwood City, CA). Supplementary anti-HEV testing was carried out at Genelabs (San Antonio, TX) by immunoassay using the *E. coli*-derived SG-3 protein (327 carboxy-terminal amino acids of HEV-ORF2 of the B strain) with samples diluted 1:100. The performance of this assay has previously been described in detail [Yarbough et al., 1994]; around 2% US unpaid blood donors and 3% US hemophiliacs have anti-SG-3 antibodies. IgM anti-HEV was assayed at Genelabs (San Antonio, TX), at 1:100 dilution, using the same antigens as in the IgG anti-HEV test described previously [Yarbough et al., 1994]. HEV RNA was tested in RNA extracted from 100 μl of serum by the guanidinium acid-phenol method and assayed by RT-PCR, with outer primer pairs HEV-3043 (antisense) and HEV-3044 (sense), and inner primer pairs HEV-1 and HEV-3 (a kind gift from S. Jameel) derived from HEV-ORF1, as previously described [Jameel et al., 1992]. This was followed by Southern blot hybridization with oligonucleotide probe DET-3, 5′end-labelled with gamma-^{32}P ATP. A cDNA fragment consisting of a PCR amplified product of 343 bp was included as a positive control.

Statistical Analysis

The Chi square test (or Fisher's exact test where applicable) was used for statistical comparisons.

RESULTS

IgG anti-HEV testing by assay no. 1 resulted in 62 initially reactive samples, although, upon retesting, only 47/101 (46.5%) cases were repeatedly positive. The majority of the non-repeats had absorbance values close to the cut-off value of the assay, but above the 10% grey zone recommended by the supplier. This result suggests that the cut-off level of the assay might be modified in order to avoid non-reproducible results. Absorbance values in antibody-positive samples ranged from 0.37 to 1.32 units (cut-off = 0.38 ± 0.08, average of three separate runs). In contrast, only four samples tested by assay no. 2 were reactive for IgG anti-HEV (absorbance values between 0.56 and 1.72, cut-off = 0.52 ± 0.01, average of three separate runs). These four samples were also reactive by assay no. 1. Individual absorbance values in these assays, according to the etiology of acute viral hepatitis, are as shown in Figure 1 (panels A and B, respectively).

Supplemental SG-3 antibody testing revealed that only 18/47 (38.3%) reactive samples by assay no. 1, and the four samples reactive by assay no. 2, were confirmed as positive. The majority of the non-reactive samples by supplemental assay were hepatitis A cases (Fig. 1, panel C). However, there was no relationship among the frequency of IgG anti-HEV by either assay used and the etiology of the acute hepatitis episode. Of the 54 patients negative by assay no. 1, one (1.9%) did react with the SG-3 proteins.

The epidemiologic distribution of the patients was investigated as well. There was no relationship among the frequency of IgG anti-HEV by assay no. 1 and the distribution by age, sex, or the background (data not shown). In contrast, the seroprevalence of anti-HEV by supplemental SG-3 assay increased significantly with age (10/81, 12.3%, in cases aged 45 or below vs. 8/20, 40%, in those older than 45 years; $P = .011$).

On the other hand, no patient resulted positive to IgM anti-HEV by ORF2 and ORF3 assays. In addition, HEV RNA detection was performed in antibody-positive and negative cases, but none of these gave a positive result.

DISCUSSION

In this study, we investigated retrospectively the frequency of HEV infection in sequential unselected acute hepatitis cases in a developed country, using two commercially available immunoassays. Due to the discrepant results obtained in these two immunoassays, and because the incidence of IgG anti-HEV was higher than would be expected, the possibility of the occurrence of false positive results was subsequently investigated. Supplemental antibody testing reduced the frequency of IgG anti-HEV to 17%. Thus, HEV seroprevalence might be overestimated in acute viral hepatitis cases in a devel-

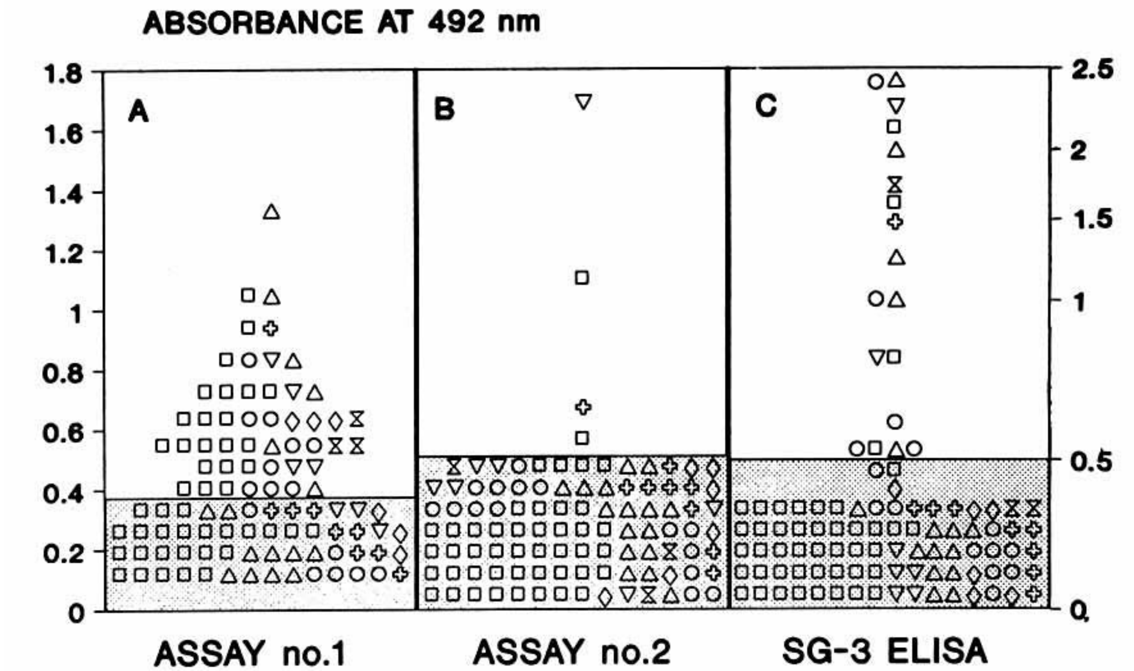

Fig. 1. Distribution of individual absorbance values in three IgG anti-HEV assays (left scale: panel A, assay no. 1, and panel B, assay no. 2; right scale: panel C, SG-3 ELISA) in 101 patients, according to the etiology of the acute hepatitis episode. Shadowed areas refer to negative values. □, HAV (n = 45); △, HBV (n = 16); ▽, HBV + HDV (n = 7); ○, HCV (n = 15); ◇, HBV + HCV (n = 6); ⧖, HBV + HDV + HCV (n = 3); ✥, NANBNC (n = 9).

oped country. It appears that the assays used in this study for the screening of IgG anti-HEV are either not specific enough (assay no. 1), or relatively insensitive (assay no. 2), at least in the population studied herein. It should be noted, however, that some of the non-confirmed specimens might have detectable antibody to ORF3 but not to ORF2 protein epitopes [Dawson et al., 1992]. Therefore, the use of additional antigens produced in bacteria, or in insect cells, and synthetic peptides [Khudyakov et al., 1993; Tsarev et al., 1993; Yarbough et al., 1994; Paul et al., 1994; Yarbough, manuscript in preparation], is helpful for elucidation of the specificity of anti-HEV antibodies. Nevertheless, we cannot rule-out completely the possibility that the high prevalence of confirmed anti-HEV be due to non-specific cross-reactivity of the anti-HEV assays.

The variability in anti-HEV results in association to the assay used may have practical implications. Thus, data obtained by supplemental assay indicate that HEV seroprevalence is age-specific in acute viral hepatitis cases, as reported previously [Lok et al., 1992]. The seroprevalence to anti-SG-3 is 1–4% in non-endemic regions and up to 16% in disease-endemic regions [Yarbough et al., 1994]. Although the 17% seroprevalence of IgG anti-HEV observed in our studied population is high, all cases with a confirmed antibody were born in developed countries (17 in Spain and one in the UK). As none of them were IgM anti-HEV positive by ORF2 and ORF3 assays, and viremia was not detected, this would suggest that HEV is not the cause of the acute illness. Whether these may be "subclinical cases," due to undetectable viremia at the time of sampling, or are persons with long-lived antibodies [Khuroo et al., 1993; Bryan et al., 1994], remains unknown. Hepatitis E may be endemic even in the absence of an epidemic report [Lok et al., 1992; Zaaijer et al., 1993], suggesting that a proportion of our patients may have had prior exposure to HEV. Where recognized risk factors for HEV, such as from travel to endemic areas (this was confirmed in only one patient in this study), are lacking, the exposure in any case might be through parenteral acquisition, or by means of an as yet unidentified source.

The development of anti-HEV to ORF2 epitopes has been shown to confer protection against reinfection by the same HEV strain, or a novo infection by a different, but immunologically cross-reactive, hepatitis E virus [Tsarev et al., 1994]. Whatever the underlying reason may be, the occurrence of false positive results should be taken into consideration in future vaccine programmes against HEV, as the HEV vaccine will be administered to seronegative individuals, and protection against HEV will be assessed by anti-HEV testing.

ACKNOWLEDGMENTS

The authors are indebted to Genelabs Technologies for providing us with the commercial HEV assays for research purposes, and especially to Patrice O. Yarbough, PhD, for the performing of supplemental tests, communication of unpublished results, and her helpful comments concerning the manuscript. We are also grateful to Shahid Jameel, PhD, of the International Centre for Genetic Engineering and Biotechnology, New Delhi,

India, for providing protocols and reagents to undertake HEV RNA detection.

REFERENCES

Bradley DW (1995): Hepatitis E virus: A brief review of the biology, molecular virology, and immunology of a novel virus. Journal of Hepatology 22(suppl.1):140–145.

Bryan JP, Tsarev SA, Iqbal M, Ticehurst J, Emerson S, Ahmed A, Duncan J, Rafiqui AR, Malik IA, Purcell RH, Legters LJ (1994): Epidemic hepatitis E in Pakistan: patterns of serologic response and evidence that antibody to hepatitis E virus protects against disease. Journal of Infectious Diseases 170:517–521.

Castillo I, Bartolomé J, Navas S, González S, Herrero M, Carreño V (1994): Virological and biochemical long-term follow-up of patients with chronic hepatitis C treated with interferon. Hepatology 19:1342–1346.

Chau KC, Dawson GJ, Mushahwar IK, Gutierrez RA, Johnson RG, Lesniewski RR, Mattson L, Weiland O (1991): IgM-antibody response to hepatitis C virus antigens in acute and chronic post-transfusion non-A, non-B hepatitis. Journal of Virological Methods 35:343–352.

Clemens JM, Taskas S, Chau K, Vallari D, Shih JWK, Alter HJ, Schleicher JB, Mimms LT (1992): IgM antibody response in acute hepatitis C viral infection. Blood 79:169–172.

Dawson GJ, Chau KH, Cabal CM, Yarbough PO, Reyes GR, Mushahwar IK (1992): Solid-phase enzyme-linked immunosorbent assay for hepatitis E virus IgG and IgM antibodies utilizing recombinant antigens and synthetic peptides. Journal of Virological Methods 38:175–186.

Farci P, Alter HJ, Wong D, Miller RH, Shih JW, Jett B, Purcell RH (1991): A long-term study of hepatitis C virus replication in non-A, non-B hepatitis. New England Journal of Medicine 325:98–104.

Huang C-C, Nguyen D, Fernandez J, Yun KY, Fry KE, Bradley DW, Tam AW, Reyes GR (1992): Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). Virology 191:550–558.

Jameel S, Durgapal H, Habibullah CM, Khuroo MS, Panda SK (1992): Enteric non-A, non-B hepatitis: epidemics, animal transmission and HEV detection by polymerase chain reaction amplification. Journal of Medical Virology 37:263–270.

Khudyakov YE, Khudyakova NS, Fields HA, Jue D, Starling C, Favorov MO, Krawczynski K, Polish L, Mast E, Margolis H (1993): Epitope mapping in proteins of hepatitis E virus. Virology 194:89–96.

Khuroo MS, Kamili S, Dar MY, Moeckli R, Jameel S (1993): Hepatitis E and long-term antibody status. Lancet 341:1335.

Lok ASF, Kwan WK, Moeckli R, Yarbough PO, Chan RT, Reyes GR, Lai CL, Chung HT, Lai TST (1992): Seroepidemiological survey of hepatitis E in Hong Kong by recombinant-based enzyme immunoassays. Lancet 340:1205–1208.

Paul DA, Knigge MF, Ritter A, Gutierrez R, Pilot-Matias T, Chau KH, Dawson GJ (1994): Determination of hepatitis E virus seroprevalence by using recombinant fusion proteins and synthetic peptides. Journal of Infectious Diseases 169:801–806.

Quiroga JA, van Binsbergen J, Wang CY, Pardo M, Navas S, Trines C, Herrero M, Carreño V (1995): Immunoglobulin M antibody to hepatitis C virus core antigen: Correlations with viral replication, histological activity and liver disease outcome. Hepatology 22:1635–1640.

Reyes GR, Purdy MA, Kim J, Luk K-C, Young LM, Fry KE, Bradley DW (1990): Isolation of a cDNA from the virus responsible for enterically transmitted non-A, non-B hepatitis. Science 247: 1335–1339.

Tam AW, Smith MM, Guerra ME, Huang C-C, Bradley DW, Fry KE, Reyes GR (1991): Hepatitis E virus (HEV): Molecular cloning and sequencing of the full-legth viral genome. Virology 185:120–131.

Tsarev SA, Emerson SU, Reyes GR, Tsareva TS, Legters LJ, Malik IA, Iqbal M, Purcell RH (1992): Characterization of a prototype strain of hepatitis E virus. Proceedings of the National Academy of Sciences of the United States of America 89:559–563.

Tsarev SA, Tsareva TS, Emerson SU, Kapikian AZ, Ticehurst J, London W, Purcell RH (1993): ELISA for antibody to hepatitis E virus (HEV) based on complete open-reading frame-2 protein expressed in insect cells: identification of HEV infection in primates. Journal of Infectious Diseases 168:369–378.

Tsarev SA, Tsareva TS, Emerson SU, Govindarajan S, Shapiro M, Gerin JL, Purcell RH (1994): Successful passive and active immunization of cynomolgus monkeys against hepatitis E. Proceedings of the National Academy of Sciences of the United States of America 91:10198–10202.

Yarbough PO, Tam AW, Fry KE, Krawczynski K, McCaustland KA, Bradley DW, Reyes GR (1991): Hepatitis E virus: Identification of type-common epitopes. Journal of Virology 65:5790–5797.

Yarbough PO, Tam AW, Gabor K, Garza E, Moeckli RA, Palings I, Simonsen C, Reyes GR (1994): Assay development of diagnostic tests for hepatitis E. In Nishioka K, Suzuki H, Mishiro S, Oda T (eds): "Viral Hepatitis and Liver Disease." Tokyo: Springer-Verlag, pp 367–370.

Zaaijer HL, Kok M, Lelie PN, Timmerman RJ, Chau K, van der Pal HJ (1993): Hepatitis E in the Netherlands. Imported and endemic. Lancet 341:286.